

UNIVERSITE DE GENEVE
Département de Médecine
Division d'Hématologie

FACULTE DE MEDECINE
Professeur P.A. MIESCHER

**GENERATION OF VIRUS SPECIFIC CYTOTOXIC ACTIVITY
IN HUMAN PERIPHERAL LYMPHOCYTES
AFTER VACCINATION
WITH VACCINIA VIRUS AND MEASLES VIRUS**

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présentée à la faculté de médecine de l'Université de Genève
pour obtenir le grade de docteur en médecine —

par

Luc Henri PERRIN
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La Faculté de médecine, sur le préavis du professeur Peter A. Miescher, autorise l'impression de la présente thèse, sans prétendre par là émettre d'opinion sur les propositions qui y sont énoncées.

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Le doyen :
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Generation of Virus-Specific Cytolytic Activity in Human Peripheral Lymphocytes after Vaccination with Vaccinia Virus and Measles Virus

Abstract. Human peripheral blood lymphocytes (PBL) harvested after vaccination with vaccinia or measles virus showed a specific activity against virus-infected target cells. This activity peaked on day 7 and was specific for the target cells infected with the virus used for the vaccination. The cytotoxic activity was not related to HLA markers. The cells involved in the cytolytic process were lymphocytes bearing Fc receptors. In addition, the cytotoxic activity was abrogated by more than 90% by rabbit Fab'2 anti-human IgG. It is therefore likely that two subpopulations of lymphocytes are involved: an antibody-secreting cell providing specific antiviral antibody and an effector cell bearing Fc receptor (K cells). Finally, these experiments suggest that antibody-dependent cell cytotoxicity may play a major role in the recovery from virus infection in man.

Introduction

The mechanism of host defence against viral infection implicates many nonspecific and specific immunologic components [1, 2].

The humoral immune response seems to play a major role in the recovery from some virus infections in man, such as poliomyelitis virus [3]. More generally, specific antibodies are a major factor in inhibiting the spread of viral infections, thus assisting recovery from a primary viral infection and prevention of reinfection by the same agent [4, 5].

The cytotoxic activity of human peripheral lymphocytes (PBL) toward virus-infected cells has been extensively studied, but there is no clear evidence that lymphocytes by themselves have a cytotoxic activity in absence of specific antiviral antibodies. In contrast, human immune or nonimmune lymphocytes have a good cytotoxic activity toward virus-infected cells in the presence of specific antiviral antibodies [6-8]. There is, however, some suggestion that a cellular immune response, similar to the response

observed in the mouse with a variety of viral infections, is important in man. For example, delayed hypersensitivity reactions can be observed in man after a variety of virus infections [9]. Moreover, children with congenital agammaglobulinemia recover without major complication after measles virus infection or vaccinia virus vaccination. In contrast, children suffering from profound T cell defects develop major complications such as giant cell pneumonia and encephalitis after measles virus infection, and vaccination of these children with vaccinia virus has led to generalized vaccinia infection [10].

We decided, therefore, to investigate whether humans can also generate a cell-mediated cytotoxic response against virus-infected cells. Similar studies carried out in mice have indicated that the peak of the cytotoxic response occurs around day 5 to day 7 and that the cytotoxic T cells lyse virus-infected cells only when both types of cell share at least one gene product of the K or D region of the major murine histocompatibility complex (H-2) [11, 12]. To allow for these elements, we chose to immunize volunteers with either vaccinia virus or measles virus and to use autologous virus-infected target cells or target cells of known HLA specificities.

Methods

Immunization of Human Subjects

Twelve normal adults (21-43 years of age) were vaccinated with vaccinia virus by scarification with smallpox vaccine in the area of the left deltoid muscle. Six children (under 5 years of age), who have never had measles virus infection, were also vaccinated with live attenuated measles virus. Blood was obtained from the antecubital vein before vaccination and at various intervals after vaccination and transferred sterilely in 50-ml plastic tubes containing 20 U of heparin/ml of blood.

Preparation of Peripheral Blood Lymphocytes

PBL were purified on ficoll hypaque gradient [13]. Monocytes were depleted by adherence to plastic dishes after incubation of the PBL suspension for 1 h at 37°C in 5% CO₂. When necessary, PBL were segregated into T-depleted PBL and T cells by a rosetting technique using sheep red blood cells treated with neuraminidase and ficoll hypaque gradient [14]. A similar technique using ox erythrocytes coated with a sub-agglutinating dose of rabbit anti-ox erythrocyte serum was used for the separation of PBL into PBL depleted of Fc-receptor-bearing lymphocytes and lymphocytes bearing Fc receptor [16].

Cell Lines

Human skin fibroblast primary cultures were raised from biopsies taken from the forearms of six of the vaccinated volunteers and processed as described [15]. Normal HeLa cells and HeLa cells persistently infected with measles virus were raised and grown as previously described [16]. Vero cells were obtained from Flow Laboratory.

Viruses

The WR strain of vaccinia virus was a gift from Dr W. K. Joklik (Duke University, Durham, N.C.) and was titered on Vero cells. Wild-type Edmonston measles virus was

obtained from the American Type Culture Collection (Rockville, Md.) passed on HeLa cells and titered on Vero cells. Preliminary experiments showed that optimal results could be obtained in the ^{51}Cr release assay by infecting target cells with vaccinia virus at a multiplicity of infection of 4, 4 h before use, and with measles virus at a multiplicity of infection of 2, 15 h before use. Using immunofluorescence, it was found that more than 95% of the target cells were infected at these times by each virus.

HLA Determination

HLA determination was performed in the laboratory of Professor Paul Terasaki (UCLA Medical School, Los Angeles, Calif.).

Antisera

Rabbit anti-ox red blood cell (ORBC) antiserum was raised by injecting rabbits intravenously with increasing amounts of 10% ORBC (0.5-3ml) twice weekly for a month. Rabbit anti-human IgG serum was raised by injecting rabbits at 3-week intervals with 1 mg of human IgG in freund's complete adjuvant for 3 months. Specific rabbit IgG anti-human IgG fractions were obtained by affinity chromatography using sepharose 4B coupled with human IgG column, and the Fab'2 fragments were obtained by pepsin digestion.

Cytolytic ^{51}Cr Release Assay

The ^{51}Cr release assay was carried out on 6-mm flat-bottomed Falcon TC 3040 micro-test plates, basically as in [15], using either 1×10^4 fibroblasts as target cells or 2×10^4 HeLa cells. Virus was added to the cells after labelling and the target cells, either infected or non-infected (used as control), were washed before use by sucking off the medium. The percent of specific ^{51}Cr released was calculated according to the following formula:

$$\frac{\text{E-S}}{\text{Max-S}} \times 100$$

Where E = ^{51}Cr released from the target cells in presence of lymphocytes; S = ^{51}Cr released in presence of medium only; and Max = ^{51}Cr released from the target cells in presence of water and 1% NP40. ^{51}Cr release was calculated separately in each condition for both infected and noninfected target cells and the release induced by PBL or lymphocyte subpopulations from uninfected target cells (0-30%) was subtracted from the values obtained with infected target cells. All tests were run in triplicate.

Results

Table 1 shows the percentage of specific ^{51}Cr released from vaccinia-infected autologous fibroblasts by immune PBL. The PBL from three volunteers were purified on different days after vaccination with vaccinia virus and assayed in an 18 h ^{51}Cr release assay. The peak of cytotoxicity occurred between day 5 and day 7 and the activity almost completely disappeared on day 30. ^{51}Cr release did not increase by more than 7% when noninfected fibroblasts were incubated with the immune PBL at the peak of the response, and these values were subtracted from the values observed

Table 1. Time course of the cytotoxic activity of human peripheral blood lymphocytes toward human autologous fibroblasts infected with vaccinia

Donors	Days after vaccination				
	0	5	7	12	30
1	5	20	52	13	7
2	-1	13	43	23	5
3	1	24	54	10	-

Human peripheral blood lymphocytes from 3 donors harvested at different days after vaccination were tested at an effector to target ratio of 20 to 1. The amount of ^{51}Cr released was determined after 18 h incubation and is expressed in the table after subtraction of values obtained for uninfected fibroblasts as the mean of triplicate determination. The SEM did not exceed 6%. Spontaneous release for both infected and uninfected fibroblasts was less than 26%

using infected fibroblasts. Similar results were obtained by using either PBL with adherent cells or PBL depleted of adherent cells by glass adherence.

In the following experiments, the immune PBL were assayed at the same time (7 days after vaccination) on autologous, homologous, and heterologous infected and noninfected target cells. As can be seen in Table 2, using the vaccinia system, the target cells were lysed by the immune PBL independently of the presence of common

Table 2. Studies on the relationship between HLA determinants on human peripheral blood lymphocytes and on vaccinia-virus-infected targets for immune specific lysis to occur^a

Specific ^{51}Cr release from infected human fibroblasts from donors (HLA-A/B)(%)						
Donors						Mouse
HLA type	(2/28/12,14)	(1,-/8,37)	(2,3/35,8)	(3,10/7,-)	(2,26/12,38)	L-929
25,28/12,39	6.9	11.3	16.5	14.3	13.9	17.5
2,28/12,14	42.3 ^b	3.5			39.0	
1-/8,37		45.0	30.5		74.0	51.0
2,3/35,8	25.8	27.9	38.1	19.3	28.7	55.6
3,10/7,-		17.8	18.9	22.9		
2,26/12,38	35.5	42.1			33.2	35.5

^a PBL harvested 7 days after vaccination with vaccinia virus were incubated with vaccinia-infected or uninfected fibroblasts of different HLA specificities or with murine L 929 cells at effector to target ratio of 12.5:1 for 18 h. Tests were run in triplicate, and the SEM did not exceed 6%, calculated as in Table 1. Within each experiment the spontaneous release was similar among the various fibroblast targets (between 20 and 32%). Spontaneous release for uninfected and infected L 929 cells was between 17-26%

^b Number refers to the mean value of the specific ^{51}Cr released from infected cells. Auto-logous combinations are in italics

antigens on effector and target cells; moreover, heterologous murine L929-infected cells were also lysed by the immune PBL. Similar observations were made using PBL purified from children 7 days after their vaccination with measles virus and tested against targets infected with measles virus (Table 3), since target cells bearing different HLA determinants were lysed to the same extent by the immune PBL.

The lytic process was specific as reported in Table 4. The immune PBL were only lytic against target cells infected with the virus used for the vaccination and not for the unrelated virus.

Table 3. Ability of peripheral blood lymphocytes of children vaccinated with measles virus to lyse HeLa cells and fibroblasts of different HLA specificities infected with measles virus

Donors	Specific ^{51}Cr release from measles-virus-infected		
	Human fibroblasts		HeLa cells
	1,-/8,37	2,28/12,14	
1	38 ± 4	27 ± 5	43 ± 6
2	21 ± 3	24 ± 4	35 ± 5
3	17 ± 2	23 ± 3	40 ± 6

Peripheral blood lymphocytes harvested 7 days after vaccination with measles virus were incubated with measles infected or uninfected fibroblasts or HeLa cells infected or not at an effector to target ratio of 12.5:1. Tests were run and calculated as in Table 1, spontaneous release for both infected and noninfected targets was less than 32%

Table 4. Specificity of the lysis of virus-infected targets by immune peripheral blood lymphocytes

Vaccination with	Donors	Specific ^{51}Cr release from target cells infected with (%)			
		Vaccinia virus		Measles virus	
		Day 0	Day 7	Day 0	Day 7
Vaccinia	1	2 ± 3	55 ± 6	10 ± 3	12 ± 3
Vaccinia	2	6 ± 2	26 ± 3	5 ± 2	4 ± 2
Vaccinia	3	1 ± 3	41 ± 4	23 ± 5	26 ± 5
Measles	4	ND	$< 1 \pm 2$	10 ± 2	38 ± 3
Measles	5	ND	3 ± 2	11 ± 3	46 ± 4
Measles	6	ND	1 ± 2	13 ± 2	54 ± 3

Peripheral blood lymphocytes from individuals vaccinated with either vaccinia virus or measles virus were tested against target cells infected with vaccinia or measles virus 7 days after vaccination at a lymphocyte to target ratio of 20:1. Tests were run and calculated as in Table 1. Results are expressed as the mean $\pm$ SEM

The next set of experiments (Table 5) was designed to identify the active lymphocyte subpopulation, using PBL from volunteers vaccinated with vaccinia virus and autologous fibroblasts infected with the same virus. The cytolytic activity was found almost entirely in the non-T cell subpopulation and, furthermore, depletion of either the total PBL population or of the non-T cell population from Fc-bearing lymphocytes almost completely abolished the lytic activity of the total PBL or non-T cells. Experiments conducted in the measles system gave similar results. PBL and lymphocyte subpopulations from two children vaccinated with measles virus were tested against homologous fibroblasts and induced the following percentage of specific release: first child - PBL 27%, non-T cells 29%, T cells 5% specific release; second child - PBL 25%, non-T cells 28%, T cells 4%.

Finally, when immune PBL from two individuals vaccinated with vaccinia virus were assayed in presence of 20 μ g of specific rabbit Fab'2 anti-human IgG on autologous infected fibroblasts, their lytic activity was decreased by more than 90% (donor 1: 4% versus 42% specific release; donor 2: 2% versus 26%).

Table 5. Role of total peripheral blood lymphocytes (PBL) and different PBL subpopulations on the lysis of human fibroblasts infected with vaccinia virus

		⁵¹ Cr release from infected human fibroblasts (%). Lymphocytes depleted of Fc ⁺ -bearing cells	
Lymphocyte population		No	Yes
Donor 1	Total PBL	32 $\pm$ 5	4 $\pm$ 3
	T	5 $\pm$ 3	4 $\pm$ 1
	T depleted PBL	47 $\pm$ 6	7 $\pm$ 3
Donor 2	Total PBL	29 $\pm$ 6	5 $\pm$ 2
	T	6 $\pm$ 3	2 $\pm$ 4
	T depleted PBL	37 $\pm$ 4	6 $\pm$ 6

Peripheral blood lymphocytes obtained 7 days after vaccination with vaccinia virus were separated into total PBL, T, or non-T subpopulations; the various populations were depleted of Fc-receptor-bearing cells (see Material and Methods). The different lymphocytes preparations with or without Fc-receptor-bearing lymphocytes were incubated with vaccinia-infected or not autologous fibroblasts at a ratio of 12.5:1. Tests were run and calculated as in Table 1

Discussion

These experiments were conducted to study in a ⁵¹Cr release assay: (1) the cytolytic activity of human peripheral lymphocytes against human target cells infected with vaccinia and measles virus after vaccination with these viruses; (2) the time course of the appearance of cytolytic activity; (3) the lymphocyte population involved in the cytotoxicity; and (4) the eventual requirement of compatibility of HLA antigens present on target and effector cells.

It was found that the PBL of individuals infected with either virus do develop a cytotoxic activity against virus-infected cells. This cytotoxic activity was specific for the targets infected with the virus used for the vaccination and peaked between day 5 and day 7 after vaccination. The subpopulation involved in the cytolytic process was composed of Fc-receptor-bearing lymphocytes. In addition, the lytic activity was not limited to autologous fibroblasts and no effective relationship could be demonstrated between HLA antigens present on target cells and effector cells. These results seem to contrast with results reported in the murine model, where spleen cells must share at least one gene product of the K and D region of the H2 complex (the analogue of the HLA system in man) with the infected target cells in order to induce the lysis of the targets [11]. However, it was shown that T cells were the cytotoxic cells in the murine system, whereas, in our human system, T cell subpopulations induce either no or only minimal lysis of infected targets. In humans, the cytotoxic cells detected after vaccination are found in the non-T cell fraction and, furthermore, depletion of lymphocytes bearing the Fc receptor abrogates the lytic activity of PBL. Other experiments showed that the lytic activity is abrogated upon addition of rabbit Fab'2 anti-human IgG to the assay, thus suggesting that human antibodies are needed for the lysis of the target cells. These two properties, nature of the effector cells (Fc-receptor-bearing lymphocytes) and dependence of antibodies, have been shown to be characteristic of the K cell system or antibody-dependent cell cytotoxicity [17]. This also suggests that in our system two different lymphocyte subpopulations are involved: a lymphocyte subpopulation acting as effector cells and an antibody-secreting cell subpopulation (B cells) providing the specific antiviral antibodies. The transient cytolytic activity detected could be related to the fact that B lymphocytes secreting actively anti-viral antibodies are present in a sufficient amount only during the peak of the immune response in the peripheral blood. This last element could be related to the detection of plasma cells in peripheral blood during acute viral infection. Experiments are in progress for the detection of specific anti-viral antibodies or for specific anti-viral antibody-secreting cells in peripheral human lymphocytes during acute viral infection.

The interest of presenting results of two different viral infections is that in the vaccinia system, the volunteers have already been in contact with the infectious agent following vaccination during their childhood; thus there is probably a secondary response. In the measles system, however, the vaccinated children have never previously been in contact with measles virus; thus we probably have a primary immune response. In both instances, the results were similar.

These experiments also show that the immune response of humans could be different to that of mice infected with the same agents, since mice infected with vaccinia virus develop cytotoxic T cells. These experiments also seem to contradict some clinical observations in humans showing that children with profound T cell defects develop major complications following measles virus infection or vaccination with smallpox virus (which is close to vaccinia virus).

When considering these arguments, the following points should be taken into account. First, in the murine system, mice are injected with a high dose of virus (approx. 10^7 PFU) by intravenous injection and they develop a systemic infection; however, in humans vaccinated by scarification with a low dose of virus (approx. 10^2 PFU),

infection is more localized and this may modulate the immune response in favor of a predominant humoral response. Secondly, in patients with a profound T cell defect, it is possible that the defect in T helper subpopulation is the major missing factor, rather than the defect in the cytotoxic effector T cell subpopulation.

It is interesting that antibody-dependent cell cytotoxicity is difficult to demonstrate, but that cytotoxic T cells are easy to demonstrate in mice during acute viral infection and that the inverse is observed in humans [6, 8, 11, 18]. This raises the question of the adaptation of the immune system during evolution.

Finally, these experiments show by an in vitro assay using PBL that the antiviral cytotoxic activity present after vaccination is antibody-dependent and cytotoxic T lymphocytes do not seem to have a major role; this does not exclude, however, the possibility that T lymphocytes may play an active role at the level of the lesion itself.

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